EXHIBIT A

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ORIGINAL ARTICLE

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Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*

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Abstract The expression of the gene coding for the carotenogenic enzyme phytoene synthase is highly regulated. To study this, its promoter and truncated versions thereof were translationally fused to the luciferase gene as a reporter and these constructs were used to transform *Arabidopsis thaliana*. The full-length promoter was shown to be active in the dark, but mediated positive responses towards different light qualities (far-red, red, blue and white light). Among the herbicides tested, norflurazon and gabaculine showed no notable effects, while CPTA abolished light induction completely. Response towards different light qualities was mediated by a TATA box-proximal promoter region up to position −300, containing G-box-like elements involved in the distinction of different monochromatic light qualities applied. This is detected in electrophoretic mobility shift assays (EMSAs), which reveal differential complex formation. A TATA box distal region of the promoter was shown to be responsible for a high basal promoter activity that was not modulated by different light qualities. Using EMSAs, a novel *cis*-acting element ATCTA occurring in tandem between positions −854 and −841 proved to be decisive in this respect. The motif was found in several other promoter regions involved in carotenoid and tocopherol biosynthesis, as well as in the promoter regions mediating the expression of photosynthesis-related genes. The functional equivalence of the motifs was shown by successfully using the respective regions in EMSAs. We conclude that the ATCTA motif represents an element capable of mediating a coordinated regulation of these pathways at the transcriptional level.

Keywords Carotenoid · Light regulation · Norflurazon · Phytochrome · Phytoene synthase

Abbreviations CPTA: 2-(*p*-chlorophenylthio)triethylammonium chloride · GBF: G-box binding factor · GGPS: geranylgeranyldiphosphate synthase · EMSA: electrophoretic mobility shift assay · PDS: phytoene desaturase · PHY: phytochrome · PSY: phytoene synthase

Introduction

The biosynthesis of carotenoids is regulated in response to both developmental and environmental stimuli, e.g. during chromoplast development in flowers and fruits or during the process of chloroplast development. In chromoplasts, carotenoids are massively accumulated to exert mainly ecological functions, while in chloroplasts a coordinated supply of carotenoids and chlorophylls is physiologically crucial. Carotenoid-free plants cannot survive in the light because in the photosynthetic apparatus carotenoids function in both the acquisition of light energy and the protection against light (Demmig-Adams et al. 1996). To perform these tasks, these pigments are localized together with chlorophyll molecules in the reaction centers of the photosystems as well as in the light-harvesting complexes.

Among several environmental stimuli regulating chloroplast development, light is the most important. Here, light is perceived differentially, e.g. by the phytochrome system and by cryptochromes, to control this process (for reviews, see Frankhauser and Chory 1997;

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Batschauer 1998; Lin 2000). Since light plays a dual role and may exert deleterious effects on the emerging photosynthetic apparatus, there is a requirement for mechanisms ensuring a protective function against light, as reflected by the quantitatively and qualitatively coordinated biosynthesis of carotenoids and chlorophylls. Accordingly, the expression of carotenogenic genes during de-etiolation must be precisely regulated and coordinated with the expression of genes for carotenoid-bearing protein complexes involved in photosynthesis, as well as with the expression of genes involved in chlorophyll biosynthesis, etc.

We investigated previously the expression of carotenogenic mRNAs during de-etiolation using quantitative RT-PCR. This revealed that transcripts of geranylgeranyldiphosphate synthase (*ggps*) and phytoene desaturase (*pds*) remained relatively constant while a significant up-regulation of phytoene synthase (*psy*) was observed during this process. It was concluded that the up-regulation of carotenoid biosynthesis relied essentially on the transcriptional activation of the *psy* gene, which codes for the first enzyme specifically committed to carotenoid biosynthesis. A detailed analysis of the light qualities mediating the light induction of *psy* revealed that the phytochrome system was involved. The responses of *phyA* and *phyB* mutants allowed us to assign a major role to PHYA; in contrast, PHYB was not involved. However, since in *phyA/phyB* double mutants increased *psy* transcript amounts were observed under red (R) and blue (B) light, the involvement of other phytochromes (in *Arabidopsis thaliana* PHYC to PHYE) and of cryptochromes could not be excluded (von Lintig et al. 1997). In an extension of these studies, it was shown later in *Sinapis alba* that posttranscriptional as well as posttranslational events, such as the formation of the prolamellar body and of competent membrane structures, are decisive in the regulation of the enzymatic activity of PSY and thus of carotenoid biosynthesis (Welsch et al. 2000).

The corresponding situation in chromoplast-bearing plants may be more complex. Taking tomato fruit as an example, the formation of large amounts of carotenoids involves at least in part a second set of genes differing in the tissue specificity of their expression. Two differentially regulated *psy* genes (*psy1* and *psy2*) as well as two lycopene ß-cyclase (*cyc-b* and *lcy-b*) genes exist here. *Psy1* plays a predominant role in the non-green tissue of fruit beyond the breaker stage, while *psy2* is involved in the carotenoid biosynthesis of green leaves. Similarly, *cyc-b* and *lcy-b* are predominantly involved in carotenoid biosynthesis in chromoplast or chloroplast-bearing tissues, respectively (for recent reviews, see van den Berg et al. 2000; Hirschberg 2001). The transcriptional regulation of the carotenoid biosynthesis genes seems light-regulated via the phytochrome system in tomato fruit (Alba et al. 2000), but the coordination with genes involved in photosynthesis, which is very strict in leaves, is lost. Thus hitherto unknown developmental factors must be involved. One further determinant of carotenoid accumulation in chromoplasts is provided by the formation of carotenoid sequestering structures such as lipid globules, crystals, membranes or proteolipid fibrils (Rabbani et al. 1998; for review see Camara et al. 1995).

To circumvent the complications given above, we selected *A. thaliana* as a system to investigate the regulation of carotenoid biosynthesis in photosynthetically active tissues. *A. thaliana* does not develop chromoplasts and *psy* is present as a single copy gene.

In the present investigation, we report on the cloning and the structural and functional analysis of the *psy* promoter to substantiate its key regulatory role in green tissues. Using transgenic *A. thaliana* lines transformed with the promoter fused to the luciferase gene as a reporter, we investigated promoter activity in different tissues, under different light qualities and in the presence of herbicides. The use of promoter truncations in combination with gel retardation assays to detect DNA-complex formation allowed the characterization of responsible *cis*-acting elements. Our results indicate a spatial separation of *cis*-acting elements mediating different light responses, as well as the existence of a novel *cis*-acting element enabling strong basal activity. This latter element was also found in a variety of different photosynthesis-related genes, indicating a possible co-regulation at the transcriptional level.

Materials and methods

Cloning of the *psy* gene

For the isolation of the *psy* gene, a genomic library of *Arabidopsis thaliana*, ecotype Wassilewskija (Schulz et al. 1994) was screened. As a probe, a PCR fragment was amplified from a plasmid carrying the *A. thaliana psy*-cDNA (GenBank accession number L25812; Bartley and Scolnik 1994) with the oligonucleotides 5′-TTG TGG GTT GGT AAG GGT TC-3′ and 5′-CGT AGA TTG CCC AAA TCG CC-3′ and radiolabelled using the Klenow fragment (Klenow and Henningsen 1970). From the isolated genomic clone, a 7-kb *Eco*RI fragment containing the *psy* gene was subcloned into pBSKM. 5′ subclones were produced using restriction sites and the exonuclease method with the enzymes *Bam*HI and *Pst*I (Henikoff 1984) and then sequenced.

Translational fusions of different promoter truncations with the luciferase gene were produced by subcloning corresponding promoter fragments into the vector pSP*luc*$^+$ (Promega, Mannheim, Germany). For binary constructs, *psy/luc* fusions were isolated and subcloned into the vector pBIN-35S-mGFP (kindly provided by Dr. M. Rodriguez), replacing the 35S-mGFP region.

Luciferase and GUS measurement

Luciferase activity in seedlings from homozygous T2 lines was determined according to Iida et al. (1995) using a luminometer (lumat LB9501, Berthold, Wildbad, Germany). Protein concentration in the supernatants was determined according to Bradford (1976). All measurements were repeated three times for each T2 line; the values represent the average of all experiments for at least two strongly expressing lines of each transformation.

Quantification of transient expression was performed according to Norris et al. (1993) using the plasmid pHGi35SGUS (kindly provided by Dr. M. Rodriguez) as control plasmid. Leaves of 3-week-old *Arabidopsis* plants were bombarded with 14 pmol of an equimolar mixture of control plasmid and test plasmid (i.e. *psy/luc* fusions in the vector pSP*luc*$^+$) using a self-made particle influence

gun (Feiner et al. 1992). After incubation for 24 h in dim light, extracts were prepared and protein concentration (Bradford 1976) and luciferase activity (Iida et al. 1995) were determined immediately. Remaining extracts were kept for 24 h at −80°C, re-centrifuged and GUS activity was measured using a fluorescence spectrometer (LS50B, Perkin Elmer, Rodgau-Jügesheim, Germany).

Electrophoretic mobility shift assay

Nuclear extracts were isolated from cotyledons of mustard seedlings according to Dignam et al. (1983) and Jensen et al. (1988). Protein concentration was performed according to Bradford (1976) and aliquots were stored at −80°C.

DNA fragments used as probes which were larger than 30 bp were end-labeled with α-^{32}PdATP or α-^{32}PdCTP. Smaller probes were prepared using partially overlapping oligonucleotides and filled-in with α-^{32}PdATP or α-^{32}PdCTP using the Klenow fragment (Klenow and Henningsen 1970). DNA fragments used as competitors were prepared by the identical procedure except that all nucleotides were supplied non-radioactive. All DNA fragments were purified by gel chromatography (MicroSpin S-200 or G-50, Amersham Pharmacia Biotech, Freiburg, Germany) and incorporation of radioactivity was quantified by scintillation counting.

Binding reactions were carried out in a final volume of 30 µl and contained 15 µl 2× binding buffer [24 mM Tris/HCl pH 7.9; 24% glycerol (v/v); 70 mM KCl; 0.14 mM EDTA; 0.95 mM PMSF, 2.15 mM DTT; 15 mM $MgCl_2$; 0.01% bromphenol blue (w/v)], 6–10 nmol probe, 2 µg poly(dIdC).poly(dIdC) (Boehringer, Mannheim, Germany), 2 µg nuclear proteins and specific competitor DNA as indicated in the figure legends. After incubation at room temperature for 10 min, the binding mixtures were loaded on 4–8% polyacrylamide gels and run in 25 mM Tris/HCl pH 8.3, 190 mM glycine and 1 mM EDTA at 4°C and 200 V. Gels were wrapped in cellophane, dried at 60°C under vacuum and autoradiographed.

Plant transformation and growth conditions

A. thaliana (ecotype Wassilewskija; Arabidopsis Biological Resource Center, The Ohio State University, Columbus, Ohio, USA) plants were transformed by vacuum infiltration (Bechtold et al. 1993) with agrobacteria strain GV3101 (Koncz and Schell 1986) containing the binary *psy* promoter/luciferase constructs (see above). Putative T1 transformants were selected on kanamycin-containing (50 µg ml^{-1}) Murashige-Skoog (MS) agar plates (1× MS salts; 0.5 g MES/KOH, pH 5.7; 0.4% phytoagar). Of the initially identified T1 transformants, 5 lines per transformation were propagated. Homozygous T2 progenies were identified by the selection pattern of the corresponding T3 progenies on kanamycin-containing MS agar plates.

Seeds of white mustard (*Sinapis alba* L., harvest 1982) used for isolation of nuclear extracts were obtained from Asgrow Company (Freiburg, Germany). Seeds from homozygous *Arabidopsis* lines were surface-sterilized, plated on MS agar plates, vernalized for 4 days at 4°C and germinated for 3 days in darkness. Mustard seedlings were germinated and grown for 3 days on moist paper in darkness. Subsequently, seedlings were illuminated for 24 h with the following light conditions: white light (W), Osram L40 W (73 lamps) + Philips TLD40 W (18 lamps), fluence rate 10.9 W/m^2; red light (R), λ_{max} = 660 nm, fluence rate 5 W/m^2; far-red (FR), λ_{max} = 730 nm, fluence rate 3 W/m^2; blue light (B), Philips TLD36 W/18 lamps, plexiglass filter 627 (Röhm & Haas, Darmstadt, Germany), λ_{max} = 436 nm, fluence rate 4.1 W/m^2.

Herbicide treatments and HPLC analysis

For herbicide-containing agar plates, autoclaved MS agar was cooled to 40°C, stock solutions of the corresponding chemicals were added and the media poured into Petri plates. The following substances were used: CPTA [2-(4-chlorophenylthio)triethylammonium chloride; synthesized according to Scheutz and Baldwin 1958]; norflurazon (SAN 9789; 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinon; Mayer et al. 1989); gabaculine (2-amino-2,3-dihydrobenzolic acid; Sigma).

Seeds from *A. thaliana* (ecotype Wassilewskija) were surface sterilized and plated on MS agar plates and herbicide-containing MS agar plates. After vernalization for 4 days at 4°C, seeds were germinated for 3 days in darkness and thereafter illuminated for 24 h with W light. Seedlings were harvested, immediately frozen in liquid nitrogen, ground to powder and resuspended in 500 µl 100 mM Tris. An aliquot of 50 µl was taken for protein determination (Bradford 1976). The remaining suspension was extracted with an equal amount of $CHCl_3$/methanol (2/1, v/v). The organic phase was separated by centrifugation (5 min, 10,000 *g*), collected and the aqueous phase was re-extracted twice with $CHCl_3$. The collected organic phases were subjected to quantitative HPLC analysis. As internal standards, lycopin (Hoffmann-LaRoche, Basel, Switzerland) was used for control seedling and seedlings treated with norflurazon and gabaculine, whereas β-carotene (Sigma, Germany) was used for CPTA-treated seedlings. The HPLC-system consisting of a C30 reverse phase column (YMC 200, CROM, Herrenberg, Germany) and a gradient system using (A) methanol/*tert*-butyl-methyl ether/H_2O (75:15:15, by volume) as the polar solvent and (B) methanol/*tert*-butyl-methyl ether (50/50, v/v) as the nonpolar solvent. The gradient profile was 100% A linear to 0% A in 100 min, then isocratic for an additional 10 min at a constant flow-rate of 1 ml/min. UV/VIS spectra were monitored by a photodiode array detector (Waters 986, Eschborn, Germany). For analysis the Millenium software package version 2.1 (Waters) was used. Products were identified by chromatographic comparison to authentic reference substances isolated from *tangerine* tomatoes (Clough and Pattenden 1979) and by their spectral characteristics.

Results

Cloning of the *psy* gene and spatial pattern of expression of the luc-reporter gene

The *psy* gene was isolated by screening a genomic library of *Arabidopsis thaliana* using a fragment of the *A. thaliana psy* cDNA (Bartley and Scolnik 1994) as a probe. This yielded a 7-kb DNA fragment which revealed by sequencing the presence of the *psy* gene as part of chromosome 5 (GenBank accession number AB005238 from bp 18150 to bp 25521, Sato et al. 1997). The fragment contained the complete transcribed region of 3.1 kb, 525 bp of the 3'-untranscribed region and 3.7 kb of the *psy* promoter region. Figure 1 shows the DNA sequence of the *psy* promoter region up to position −1746. Position + 1 represents the first nucleotide of the longest cDNA isolated. Two putative TATA motifs are localized at positions −124 and −153.

To investigate regulatory regions within the promoter, we constructed six different translational fusions using the luciferase gene as the reporter at the following sites: -1746, −1314, -910, -809, -300 and −196. The fusions −1746/+716, -1314/+716, -300/+716 and −196/+716 were cloned into the vector pBIN121 upstream of the *nos* polyadenylation signal. These constructs were used for *Agrobacterium*-mediated transformation of *A. thaliana*. Homozygous inbreds were produced from several lines of all transformants to be used for expression analysis.

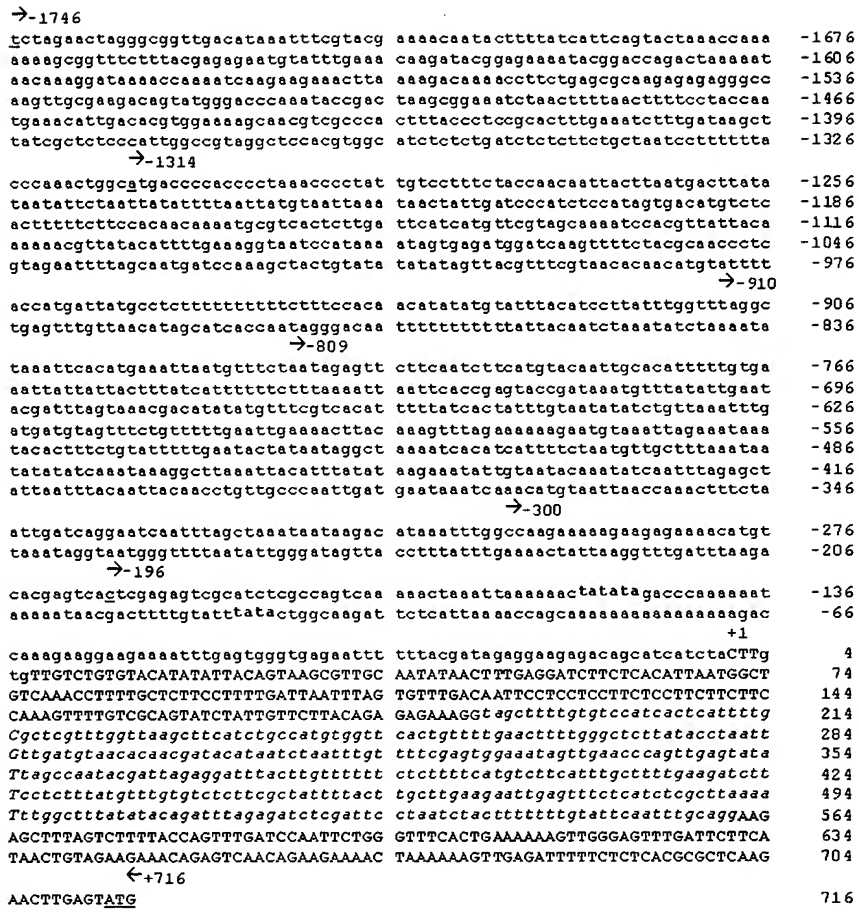

Fig. 1 Sequence of the *psy* promoter and 5′ non-translated region. Position +1 represents the first nucleotide of the longest cDNA isolated (Bartley and Scolnik 1994). Exons are shown in uppercase letters and the first intron is italicized. Breakpoints of the fusions with the *luc* reporter gene are indicated by *arrows* and *underlined letters*. Putative TATA boxes are shown in bold, the ATG start codon is underlined. The region represents the sequence from 21076 to 25521 of the PI clone MKP11 of chromosome 5 (GenBank accession number AB005238)

In plants containing the –1746/+716 *psy/luc* transgene, luciferase activity was detected in all tissues tested, including those which normally contain carotenoids only in trace amounts if any, such as roots (Fig. 2). The highest luciferase activity was measured in flowering buds and ripening seed pods while intermediate values were obtained for leaves.

Expression during photomorphogenesis

As we have shown previously, the phytochrome system mediates the light induction of PSY as observed at the transcript as well as at the protein level (von Lintig et al. 1997; Welsch et al. 2000). Using the different transgenic *psy/luc Arabidopsis* lines, we aimed at characterizing *cis*-acting elements within the *psy* promoter responsible for light induction. For this purpose, luciferase activity was determined from seedlings which were etiolated for 3 days and from etiolated seedlings illuminated for 24 h with different light qualities (Fig. 3). The longest promoter fragment used (–1746/+716) showed two- to threefold increases in luciferase activity after illumination with W, FR, R and B light. This correlates well with the increase of *psy* transcript amounts in *Arabidopsis* seedlings subjected to the same light treatment (von Lintig et al. 1997).

Transformants carrying the –1314/+716 *psy/luc* fusion or the –1746/+716 *psy/luc* fusion were almost indistinguishable in their patterns of luciferase activity. However, further truncation up to position –300 led to a decrease of 20–30% in promoter activity under all light conditions. Further truncation up to –196 abolished the induction under R light completely, whereas an induction under W, FR and B light was still observed. This indicates the existence of spatially separated *cis*-acting elements responsible for R and FR/B responses: *cis*-acting elements located between –300 and –196 are essential for responses to R light while those for FR and B light response are located in the proximity of the TATA box, up to position –196. Furthermore, since both the –300/+716 and –196/+716 *psy/luc* fusions show the same luciferase activity in etiolated seedlings, the responsible *cis*-acting elements are also localized in the proximity of the TATA box region, up to –196.

Thus, two regions within the *psy* promoter appear to be mainly involved in the regulation, one being located between –1314 and –300 (heretofore TATA box distal region) responsible for a basal strong promoter activity, the other being located between –300 and the TATA box (heretofore TATA box proximal region). This latter region is mainly involved in the differentiated response towards different light qualities.

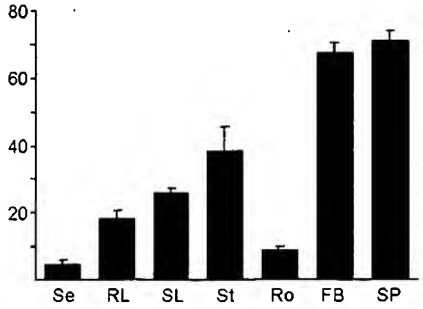

Fig. 2 Activity of the −1746/+716-*psy/luc* fusion in tissue extracts from 6-week-old transgenic *Arabidopsis* plants. Luciferase activity was determined by a luminometric assay and is expressed in relative light units (RLU) s^{-1} μg^{-1} protein (*FB* flowering buds, *RL* rosette leaves, *Ro* roots, *Se* seeds, *SL* stem leaves, *SP* seed pods, *St* stem)

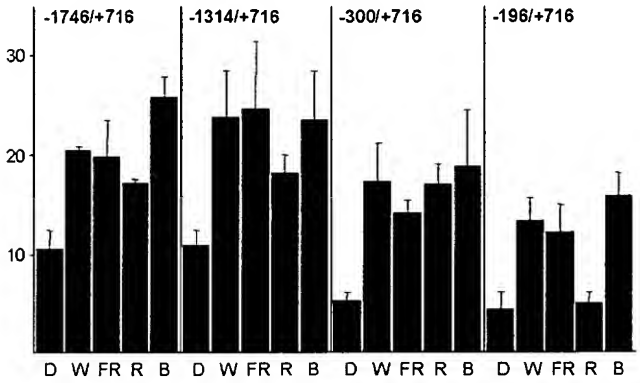

Fig. 3 Activity of different *psy/luc* fusions in transgenic *Arabidopsis* seedlings during de-etiolation. The numbers indicate the breakpoints of the *psy* promoter region fused with the *luc* gene. Transgenic *Arabidopsis* seedlings were etiolated for 3 days (*D*) and thereafter illuminated for 24 h with white (*W*), far-red (*FR*), red (*R*) and blue (*B*) light. Luciferase activity was measured luminometrically and expressed in RLU s^{-1} μg^{-1} protein

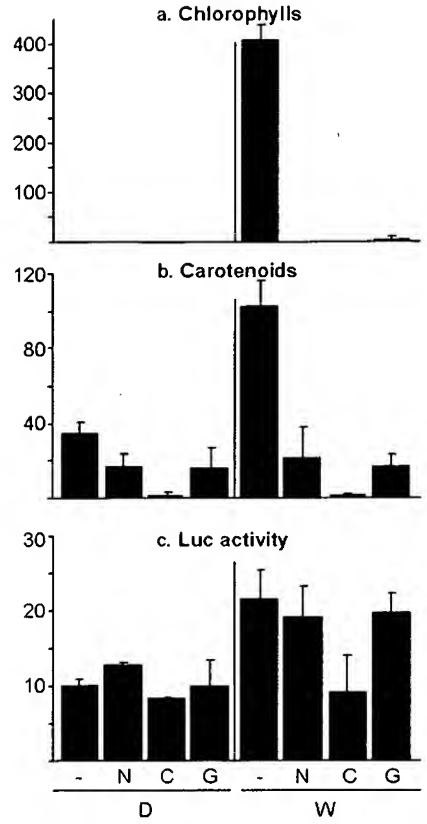

Fig. 4a–c Effects of herbicide treatments on carotenoid and chlorophyll content and *psy* promoter activity in *Arabidopsis* seedlings. Wt and transgenic −1746/+716 *psy/luc Arabidopsis* seedlings were etiolated for 3 days (*D*) and thereafter illuminated for 24 h with white (*W*) light. Chlorophyll (**a**), carotenoid content (**b**) (ng μg^{-1} protein) and c luciferase activity (RLU s^{-1} μg^{-1} protein) are shown. Data are means ± SE of two experiments except for *c* where data are means of six experiments (*C* CPTA, *G* gabaculine, *N* norflurazon)

Effects of herbicides on the expression

Herbicides that interfere with carotenoid formation have been considered to affect the regulation of carotenoid biosynthesis in a sort of feedback mechanism (Corona et al. 1996; Al-Babili et al. 1999). Therefore, the influence of different herbicides on carotenoid content and on luciferase activity in herbicide treated *psy/luc* plants was analyzed (Fig. 4). To include possible effects of herbicides on light induction, the herbicide treatment was carried out with etiolated seedlings as well as with seedlings illuminated for 24 h with W light. The two compounds acting on carotenogenic enzymes were norflurazon, which inhibits PDS and leads to phytoene-accumulation, and CPTA, which inhibits lycopene cyclase and yields lycopene. Furthermore, to compare the effects of gabaculine on the *pds* promoter reported in the literature (Corona et al. 1996), we included this compound into our studies. Gabaculine is an inhibitor of chlorophyll biosynthesis acting at the level of the enzyme glutamate 1-semialdehyde aminotransferase (Werck-Reichhart et al. 1988).

As determined by quantitative HPLC analysis, in etiolated seedlings treated with norflurazon and gabaculine, the carotenoid content reached only half of the amount present in the untreated controls (Fig. 4b). Furthermore, the carotenoid content remained constant after illumination in herbicide-treated seedlings, whereas a triplication of carotenoids occurred in untreated seedlings. In seedlings grown on CPTA, the carotenoid amount was further decreased and consisted of only traces of lycopene. As with all other herbicides tested, this remained unchanged after illumination. Thus, in this system, all herbicides used led to a decrease in carotenoid content and a complete loss of light-induced accumulation.

This loss, however, is not related in all cases to equivalent responses in promoter activity (Fig. 4c). Interestingly, the decrease in the carotenoid content affected by norflurazon and gabaculine in the dark did

not significantly change the luciferase activity in −1746/+716 *psy/luc* plants. This indicates that, contrary to that observed for the tomato *pds* promoter (Corona et al. 1996), there was no regulatory feedback mechanism acting in the presence of these two compounds. However, the regulation of signal transduction pathways leading to light induction of the *psy* promoter occurred undisturbed, since an increase in luciferase activity was observed in the respective illuminated seedlings.

CPTA treatment did not change reporter activity in dark-grown seedlings but, in contrast to norflurazon and gabaculine treatment, it abolished light induction almost absolutely.

Characterization of *cis*-acting elements of the TATA box proximal promoter region

The analysis of luciferase expression of the *psy/luc* fusions led to the conclusion that *cis*-acting elements mediating differential light responses are located within the TATA box proximal promoter region up to position −300. To further characterize these elements, EMSAs were performed using nuclear extracts from *Sinapis alba* seedlings. Using the region from −316 to −166 as a probe, the formation of specific protein/DNA complexes was observed, as shown by successful competition of complex formation with the same unlabelled probe (Fig. 5c, left). As the competition occurred also with a shorter promoter fragment corresponding to position −210 to −179, it was concluded that the corresponding *cis*-acting elements are located within this region. This finding was confirmed by EMSAs using this promoter sequence as a probe (Fig. 5c, middle). Furthermore, the formation of protein/DNA complexes correlated with the promoter activities observed under different light conditions. Extracts from seedlings illuminated with W, FR and B light showed higher binding activity than nuclear extracts from etiolated seedlings. However, when obtained from R-light-illuminated seedlings, only weak protein/DNA complexes were formed.

Although motifs of well-known plant transcription factors were not found within this −210 to −179 promoter region, two short motifs share some similarity to G-box motifs, which are known to be involved in the light regulation of several genes (in the following G1 and G2, see Fig. 5b; Giuliano et al. 1988). According to Schindler et al. (1992), mutations in these motifs affect the affinity towards GBFs. Therefore, we investigated possible functions of these two motifs by analyzing the binding activities of DNA fragments successively mutated in these motifs (Fig. 5c, right). The mutation in motif G1 led to the appearance of an additional protein/DNA complex with different migration behavior, whereas the mutation in G2 prevented complex formation completely. Thus, both motifs are necessary for the formation of the protein/DNA complex, but to a different extent: while G2 is essential for the formation, G1 contributes more to complex stability. This may be reflected in the fact that the −196/+716 *psy/luc* fusion, which includes G2, but not G1, shows slightly lower light induction than the −300/+716 *psy/luc* fusion and has lost R induction.

The fact that mutations in both motifs effected complex formation indicated the involvement of GBFs. A further clue was obtained by a competition assay us-

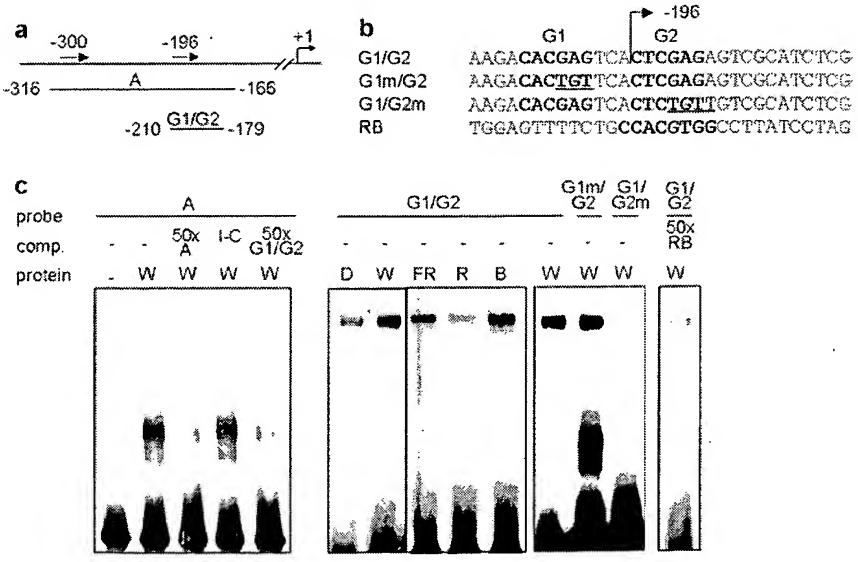

Fig. 5 **a** Fragments from the TATA box proximal region of the *psy* promoter used in electrophoretic mobility shift assays (EMSA). The numbers depict the distance in bp to the transcription start site (+1). **b** Sequences of the oligonucleotides used for the EMSAs. G-box-like elements and the G-box of the *rbcS3B* promoter are shown in bold face; mutations are given as underlined. *G1/G2* is the *psy* promoter region between −210 and −179; *RB* is the *rbcS3B* promoter region between 8212 and 8242 (Dedonder et al. 1993). **c** EMSAs with mustard nuclear extracts from etiolated (*D*) and far-red (*FR*), red (*R*), blue (*B*) and white (*W*) light illuminated mustard seedlings. Competitions were performed with the molar excess of unlabelled fragments as given [*I-C* 250 ng poly(dIdC) was added to the incubation]

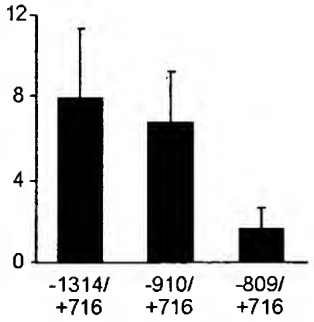

Fig. 6 Quantification of luciferase activity in *A. thaliana* leaves transiently expressing *psy/luc* fusions. The fusions used as test plasmids are indicated on the abscissa; the vector pHGi35SGUS was used as control plasmid. Equimolar amounts of test and control plasmid were used for each measurement. Luciferase activity was measured luminometrically and normalized to the fluorimetrically determined GUS activity. Data are means ± SE of three experiments

ing the perfect palindromic G-box of the rubisco SSU 3b promoter (*rbcS3b*; GenBank accession number X14564, Dedonder et al. 1993) as competitor. In this case, the complex formed with the *psy* promoter region from –210 to –179 disappeared almost completely.

Characterization of *cis*-acting elements of the TATA box distal promoter region

Beside the TATA box proximal promoter region, which mediates a differential response towards different light qualities, further *cis*-acting elements located between –1314 and –300 are responsible for a strong basal i.e. light quality-independent promoter activity (see above). To further define the position of these elements, we analyzed the transient luciferase expression of additional *psy/luc* fusions in green leaves from adult plants (grown under 8 h D/16 h W conditions). In this assay, for each test construct containing the *psy/luc* fusion, equimolar amounts of a control plasmid mediating constitutive expression of GUS (driven by the CaMV-35S promoter) were co-transformed. This allowed quantification of the luciferase values relative to the fluorimetrically obtained values for GUS activity, correcting for different transformation rates and/or tissue conditions (Norris et al. 1993). As shown in Fig. 6, the *psy/luc* fusion –910/+716 mediated almost the same promoter activity as –1314/+716 (which had been analyzed in stabile transformations). However, a strong decrease in luciferase activity was observed for the –809/+716 *psy/luc* fusion. Therefore, *cis*-acting elements responsible for the strong basal promoter activity are located between –910 and –809.

EMSAs were conducted again in order to identify *cis*-acting elements within this region using nuclear extracts from mustard seedlings (Fig. 7). Using the region –946 to –754 of the *psy* promoter as radiolabelled probe, the formation of protein/DNA complexes was observed with extracts from etiolated seedlings illuminated for 24 h with W light. The specificity of this complex was demonstrated by competition with the same unlabelled probe. Furthermore, the complex formed could also be competed with the promoter region from –856 to –825, indicating the presence of *cis*-acting elements within this region.

Using this 31-bp fragment as a radiolabelled probe, EMSAs with nuclear extracts isolated from differentially illuminated seedlings revealed that the binding activity of the corresponding *trans*-acting factors increased under all light conditions examined, compared with experiments conducted with extracts from etiolated seedlings (Fig. 7c, right). Thus, this region is indeed light responsive but does not confer differentiated responses towards different light qualities.

Although no consensus motifs from known plant transcription factors were detected in the promoter sequence investigated, the tandem occurrence of the

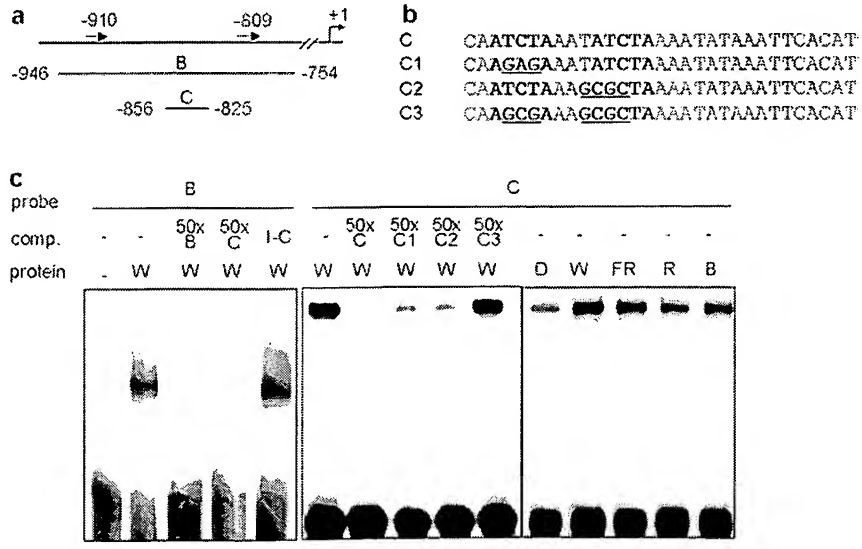

Fig. 7 **a** Fragments from the TATA box distal region of the *psy* promoter used in EMSAs. The numbers depict the distance in bp to the transcription start site (+1). **b** Sequences of the oligonucleotides used for the EMSAs. ATCTA elements are shown in bold; mutations are given as underlined. **c** EMSAs with mustard nuclear extracts from etiolated seedlings (*D*) or etiolated seedlings illuminated with white (*W*), far-red (*FR*), red (*R*) and blue (*B*) light. Competitions were performed with 50× molar excess of unlabelled fragments indicated [*I-C* 250 ng poly(dIdC) was added to the incubation]

sequence ATCTA seemed indicative (Fig. 7b). Therefore, we investigated the possible involvement of the resulting sequence motifs on complex formation. Both motifs were successively mutated and these fragments were used as competitors. As shown in Fig. 7c (middle), individual mutation of the first and of the second ATCTA motif both led to a decrease in binding activity, while simultaneous mutations in both motifs completely abolished binding ability. Therefore, the sequence ATCTA represents at least in part the *cis*-acting element for a transcription factor mediating a strong *psy* promoter activity.

To examine the distribution of this motif, we screened the promoter regions of genes from different organisms expected to be expressed coordinately with carotenoid biosynthesis during the formation of photosynthetic complexes (see Table 1). Interestingly, the ATCTA motif was found not only in promoters driving the expression of proteins involved in the carotenoid pathway, like deoxy-xylulose-phosphate synthase and PDS, but it occurred also in genes in the tocopherol biosynthesis pathway, such as for hydroxyphenyl-pyruvate dioxygenase, geranylgeranyl-diphosphate reductase, prenyl transferase and γ-tocopheryl methyltransferase. Furthermore, it was found in promoters of genes involved in photosynthesis, such as the ones coding for the chlorophyll a/b binding protein (CAB) and for plastocyanin (PC).

In order to investigate whether these ATCTA-containing promoter regions also lead to complex formation, EMSAs were conducted using nuclear extracts from mustard seedlings illuminated with W light. Promoter regions from the following genes were used as radiolabelled probes: *pds* from *Lycopersicon esculentum* (GenBank accession number U46919), *cab* from *S. alba* (GenBank accession number X16436) and *A. thaliana* (GenBank accession number J04098) and *pc* from *Pisum sativum* (GenBank accession number X16082). Consistent with the competition assays using promoter fragments mutated in one of the two ATCTA elements, these studies revealed that the tandem arrangement of the ATCTA motif was not necessary to allow complex formation. Among the promoters investigated, such an arrangement is observed only in the *cab*/*S. alba* and in the *pc*/*P. sativum* promoters, while occurring only singly in the others (see also Table 1). Fig. 8 shows the formation of protein/DNA complexes for all promoter regions tested. Their electrophoretic mobility corresponded to that observed with the probe from the *psy* promoter but differing somewhat in the amounts of retarded radiolabelled probe. The complex formed with the ATCTA motif-containing region from the *cab* promoter of *S. alba* can be competed by the corresponding ATCTA motif-containing region from the *psy* promoter, suggesting the involvement of the same transcription factor.

Discussion

The formation of a functional photosynthetic apparatus requires the synthesis of carotenoids to be well

Table 1 The ATCTA element in different promoters of plastid-localized proteins. The position of the ATCTA element is denoted relative to translational (*Tl*) or transcriptional start (*Tx*) of the gene. *Numbers in bold* indicate ATCTA elements of sequences which were used as radiolabelled probes for EMSAs in Fig. 8. GenBank accession numbers for the promoter sequences analyzed are as follows: DXS, AL161542; PSY, Ab005238; PDS (*Lycopersicon esculentum*), U46919; PDS (*Zea mays*), AF039585; CAB (*Sinapis alba*), X16436; CAB1 (*Arabidopsis thaliana*), J04098; PC (*Pisum sativum*), X16082; PC (*Arabidopsis thaliana*), S67901; PC (*Hordeum vulgare*), Z28347; HPD, NC_003070; GGR, AC011765; PT, AC007651; TMT, AC006193 (*CAB* chlorophyll a/b-binding protein, *DXS* deoxy-xylulose-phosphate synthase, *GGR* geranyl-geranyl-diphosphate reductase, *HPD* hydroxyphenyl-pyruvate dioxygenase, *PC* plastocyanin, *PDS* phytoene desaturase, *PT* prenyl transferase, *TMT* γ-tocopherol methyltransferase)

Proteins	Gene	ATCTA element	Sequence
Carotenoid biosynthesis	*dxs* (*A. thaliana*)	−819Tl	ATTTTTCTTGTAAAACATCTAAAAATTAT
		−418Tl	AAATAATATCATCAATATCTATCCAAAAC
	psy (*A. thaliana*)	**−841Tx**	TTATTACAATCTAAATATCTAAAATATAA
	pds (*L. esculentum*)	**−871Tx**	TGTTTGGAGTTTATTTATCTAAAGTAAAC
	pds (*Z. mays*)	−327Tx	AATAAACTCATTAATTATCTAAAACGAAT
		−185Tx	CTATATACTGTTCTATATCTATATTTAAT
Photosynthesis	*cab* (*S. alba*)	−1159Tl	TAATCTAAATCGAAATATCTAAATGTTTA
		−1132Tl	TACTCTAAATCTACGGATCTAATACTCAG
		−340Tl	AATGTGTTAACTAGATATCTATCGTCTCA
	cab1 (*A. thaliana*)	−490Tl	TAAATTTTATAGTTTTATCTACTTTGTTC
		−248Tl	TGAAACGCACCTAGATATCTAAAACACAT
	pc (*P. sativum*)	**−101Tx**	GTGGCACATCTACATTATCTAAATCACAT
	pc (*A. thaliana*)	−1159Tx	TTTTATAAGATAATGTATCTAGGTTTGCT
		−149Tx	TGTTCAAAGTCTCCTTATCTACTTATGCA
	pc (*H. vulgare*)	−499Tx	AATAACTGCAATTTTTATCTAAAACAATA
Tocopherol biosynthesis	*hpd* (*A. thaliana*)	−894Tl	ATACATGTAGACCAAAATCTAAAGTGTTT
		−400Tl	TCATGGCACATAGAATATCTAAGAAACTG
	ggr (*A. thaliana*)	−1001Tl	TTCTCTTACTAAAAATATCTAAAATCATA
		−124Tl	GGAAATCTCCAACAATATCTAATCCACTA
	pt (*A. thaliana*)	−908Tl	TATGAAACAAATTAAAATCTAGAAATTTC
		−151Tl	AGTACCATTCACAAGTATCTAAAAAATTG
	tmt (*A. thaliana*)	−111Tl	GAGTGAAATGATATTTATCTAAAACAAAT

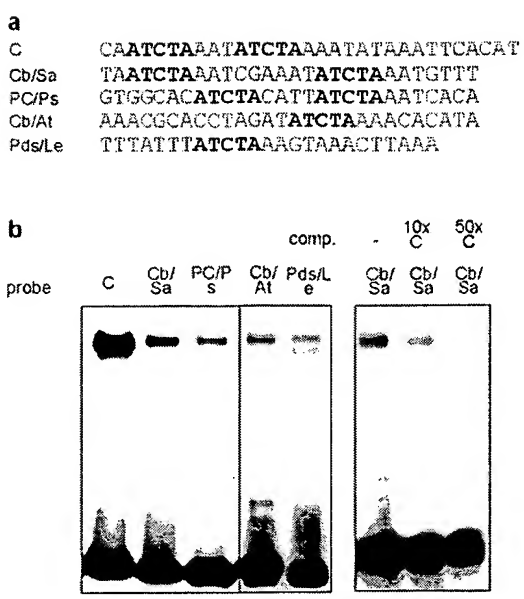

Fig. 8 a Sequences of the oligonucleotides used in the EMSAs. ATCTA elements shown in bold (*C psy* promoter region between −856 and −825, *Cb/Sa cab* promoter/*Sinapis alba, PC/Ps* plastocyanin promoter/*Pisum sativum, Cb/At cab1* promoter/*A. thaliana, Pds/Le pds* promoter/*Lycopersicon esculentum*). For GenBank accession numbers and positions within the promoters, see Table 1. **b** EMSAs with labeled oligonucleotides indicated in *A* and nuclear extracts from mustard seedlings illuminated with white light. Competitions were performed with 10× and 50× molar excess of unlabelled fragment C

coordinated with all other constituents involved in photosynthesis. The enzyme phytoene synthase (PSY) catalyzes the first reaction specifically devoted to carotenoid formation. Therefore, the finding that PSY represents the first light-induced step in the pathway on the transcriptional, protein and enzymatic level appears indicative (von Lintig et al. 1997; Welsch et al. 2000). As a consequence, we decided to focus on the *psy* promoter from *Arabidopsis thaliana* and analyzed transgenic plants carrying promoter/luciferase fusions.

The activity of the *psy* promoter observed in the dark correlates well with the PSY accumulation in the prolamellar bodies of etioplasts. Illumination with W light for 24 h induces a doubling of PSY protein (Welsch et al. 2000); concomitantly *psy* promoter activity doubles, as we show here (Fig. 3). This parallelism in transcript and protein levels indicates that translation is not involved in controlling *psy* expression. However, post-translational mechanisms are involved: it must be kept in mind that the increased promoter activity observed under FR light, for instance, yields an enzymatically inactive protein requiring the decay of the prolamellar body and the development of thylakoid membranes for activity. In one interpretation, both *psy* promoter activity in the dark and enzymatic activation by light-induced formation of membrane structures represent a synergistic mechanism to guarantee a rapid supply of photoprotective carotenoids during the initial phase of photomorphogenesis.

Nonetheless, with the results presented it can be stated that regulation of carotenoid biosynthesis is subjected to strong transcriptional regulation. This supports several studies of the steady-state concentrations of carotenogenic mRNAs during developmental processes in different systems (reviewed in Hirschberg et al. 1997).

In adult plants, the pattern of tissue-specific promoter activity did not meet expectations in all cases (Fig. 2). For example, the signal obtained for roots is surprising but may be related to a basal level of carotenoid biosynthesis. Carotenoids produced here may represent abscisic acid (ABA) precursors subjected to dioxygenase-catalyzed cleavage (Schwartz et al. 1997); in fact, ABA synthesis occurs also in roots (Milborrow 2001). The high level of *psy* promoter activity observed in developing seed pods may also be linked with ABA formation, as this phytohormone is known to prevent premature germination.

Inflorescences showed a high expression of the *psy/luc* transgene. But, while separate analysis of flower tissues is difficult in *Arabidopsis*, there are reports from tomato and tobacco expressing *gus* under control of the *pds* promoter. Here, anthers showed the strongest expression of the *pds/gus* transgene (Corona et al. 1996), followed by corollae and pistils. High expression in anthers was related to a structural function of carotenoids discussed previously (Brooks and Shaw 1968).

Carotenoid biosynthesis takes place exclusively in plastids, catalyzed by enzymes that are encoded by nuclear genes. It is well known that inhibition of plastid development may affect the expression of such proteins. For instance, plastid defects induced by norflurazon lead to differential alterations in the expression of the nuclear-encoded plastid proteins in barley seedlings (Batschauer et al. 1986). Therefore, we studied the effects of norflurazon, CPTA and gabaculine on the expression of the reporter gene. Norflurazon, a classical bleaching herbicide, and gabaculine, an inhibitor of chlorophyll biosynthesis, did not affect *psy* promoter activity during etiolation or after illumination (Fig. 4). This is quite surprising, at least in the case of norflurazon, since increased activity would be expected as a result of the photo-oxidative damage it exerts. Thus, neither the accumulation of phytoene precursors nor the reduction of final carotenoid products seems to exert notable feedback regulatory activity. In this respect, regulation of the *psy* promoter and the *pds* promoter from tomato differ; for the latter, an increase in promoter activity was reported in response to these two compounds (Corona et al. 1996). These results agree with the absence of significant effects of norflurazon treatment on the steady-state levels of the tomato *psy* mRNA (Giuliano et al. 1993). Similarly, the lycopene cyclase inhibitor CPTA, leading to lycopene accumulation, was reported to mediate a positive *pds* promoter response in the dark and in the light, whereas in our experiments the *psy* promoter did not respond to CPTA in the dark. Interestingly, CPTA treatment completely abolished the ability of this

promoter to respond positively to light. It remains to be clarified whether this is due to feedback-signaling initiated by the accumulated lycopene. Such a role for lycopene can be concluded from the unexpected results obtained with rice endosperm expressing carotenoid biosynthetic enzymes. Here, formation of *trans*-lycopene, mediated by *psy* and a bacterial *crtI* (a *trans*-lycopene forming carotene desaturase) led to the establishment of the entire carotenogenic pathway including the formation of xanthophylls (Ye et al. 1999). Similarly, expression of *crtI* in tomatoes did not lead to increased synthesis of lycopene, but to an increase in β-carotene. Endogenous carotenoid genes were concurrently up-regulated, except for *psy*, which was repressed (Romer et al. 2000). This downregulation of *psy* correlates with the effect of *trans*-lycopene on the *psy* promoter demonstrated here but conflicts with the results of CPTA experiments with daffodil flowers, where lycopene accumulation increased the abundance of *psy* transcripts and protein (Al-Babili et al. 1999).

Light induces the accumulation of carotenoids. At the level of the *psy* promoter, all light qualities tested in the present investigation increased *psy* promoter-mediated luciferase activity in transgenic seedlings carrying the −1746/+716 *psy/luc* fusion (Fig. 3). The strongest induction was observed for B light, which indicates a possible important contribution by cryptochromes. The phytochrome system is also involved: analysis of *phyA* and *phyB* mutants of *Arabidopsis* demonstrated earlier that PHYA is involved in the FR induction of *psy* transcript amounts (von Lintig et al. 1997). In agreement with these findings, FR light led to a strong increase in promoter activity. A similar response pattern toward different light qualities, albeit to a lower extent than the −1746/+716 *psy/luc* fusion, is mediated by the −300/+716 *psy/luc* fusion. However, truncation of 104 bp leading to the −196/+716 *psy/luc* fusion abolished the induction in R light completely. This indicates that *cis*-acting elements involved in the phytochrome response under R light are located between −300 and −196 of the promoter, whereas elements mediating responses to FR and B light as well as a residual promoter activity in the dark are located in the first 196 bp. Within the −300 to −196 promoter region, the position of two spatially separable *cis*-acting elements could be restricted to between positions −210 and −179 (see Fig. 5). The amounts of protein/DNA complexes formed with this region using nuclear extracts from etiolated seedlings and from FR, B and W light illuminated seedlings corresponded to the *psy* promoter activity measured under these conditions. Therefore, the binding activities of the *trans*-acting factors involved here are regulated by FR- and B-light-receptor mediated mechanisms.

The induction under R light seems to involve different *cis*-acting elements. Under R light, only weak complex formation was detectable using the −210/−179 promoter sequence as a probe. Consistent with this finding, the −196/+716 *psy/luc* fusion, which disjoins the two *cis*-acting elements characterized, lost the R induction. Therefore, additional *cis*-acting elements downstream of position −179 are required to explain the observed strong R induction in the −300//+716 *psy/luc* fusion.

The most intensely investigated group of transcription factors involved in light regulation are the GBFs (Giuliano et al. 1988; Menkens et al. 1995). A competition assay with the perfect palindromic G-box of the *rbcS3b* promoter of *A. thaliana* demonstrated the involvement of GBFs in the protein/DNA complexes formed within this *psy* promoter region (Fig. 5). Known mechanisms involved in increased GBF binding activity, such as light-induced translocation from the cytoplasm (Harter et al. 1994) or phosphorylation (Klimczak et al. 1992, 1995), may be responsible for the effects observed.

The *psy* promoter region from −215 to −166 contains two G-box-like motifs (G1 and G2). As shown by EMSAs with probes carrying mutations in G1 and G2, both motifs are involved in the formation of the protein/DNA complex observed (Fig. 5). Since GBFs belong to the group of basic leucine zipper transcription factors, it is generally assumed that a central ACGT core is necessary for binding (Foster et al. 1994) while flanking sequences determine sequence specificity (Williams et al. 1992; Izawa et al. 1993). The G-box-like motifs in the *psy* promoter do not meet this criteria, because they contain the sequences ACGA and TCGA in G1 and G2, respectively. However, there are some indications that a central ACGT core is not obligatory (de Pater et al. 1994; Yunes et al. 1994). The exact identity of the GBFs involved in the binding to *psy* promoter G1 and G2 remains to be investigated.

The light-dependent activity mediated by the −300/+716 promoter region is not qualitatively but quantitatively modulated by *cis*-acting elements located further upstream, as reflected by the strong luciferase activity in the −1314/+716 *psy/luc* plants. Quantification of transient luciferase expression revealed these elements to be located between positions −910 and −809 (Fig. 6). Competition EMSAs with nuclear extracts from mustard seedlings illuminated with W light restricts localization of the corresponding *cis*-acting elements to a region between −856 and −825. The amounts of protein/DNA complexes formed with this promoter region were not affected markedly by nuclear extracts from differently illuminated mustard seedlings (Fig. 7). Thus, the corresponding *trans*-acting factors are constitutively required for a high basal level of *psy* promoter activity.

The sequence of the DNA fragments used in these EMSAs contained two ATCTA motifs in tandem. Competition EMSAs with DNA fragments carrying the mutated motifs demonstrated that these boxes indeed bind a *trans*-acting factor (Fig. 7) and that the existence of only one ATCTA motif is sufficient. Mutation of only one motif was insufficient to prevent complex formation, while mutation of both led to a complete loss of binding capacity.

Apart from in the *psy* promoter, the ATCTA motif also occurs in promoters of genes involved in carotenoid

and tocopherol biosynthesis, and also in some photosynthesis-related genes (see Table 1). This may indicate a more general importance of this motif in the co-regulation of these genes. This is further corroborated by the observation that different ATCTA-containing promoter regions in EMSAs resulted in protein/DNA complexes with identical migration behavior but in different amounts (Fig. 8). The ATCTA element characterized here shares some similarity to a *cis*-acting element found in several *cab* genes which are recognized by CCA1. This myb-related transcription factor was shown to be involved in the phytochrome induction of these genes (Wang et al. 1997; Wang and Tobin 1998). However, footprint analysis defined the sequence $AA^{A}/_{C}AATCT$ as the binding sequence for CCA1, whereas the presence of A residues upstream of the ATCTA element is not necessary for binding of the transcription factor involved here. This can be concluded both from the competition assay using mutated ATCTA sequences (Fig. 7c), as well as from the sequences neighboring the ATCTA elements found in other promoters (Fig. 8). Therefore, the involvement of an as yet unknown transcription factor seems probable.

Work is in progress towards the molecular identification of the corresponding transcription factor, to elucidate its potential for the synergistic regulation of different photosynthesis-related biochemical pathways.

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